Application of Circular Dichroism and Infrared Spectroscopy to the Conformation of Proteins in Solution

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Circular dichroism measurements have been carried out between 185 and 250 mμ on synthetic polypeptides in various conformations: α-helix, antiparallel chain β, disordered, polyproline I, polyproline II. The characteristic bands obtained have been applied to the analysis of protein conformation in aqueous solution. Infrared absorption spectra in the amide I band region have been obtained on polypeptides and proteins in the α-helical, antiparallel chain β- and unordered conformations in H_2O, D_2O, Nujol suspensions and films. Band shifts with changing structure and solvent have been analysed in terms of the Miyazawa–Krimm theory and the results applied to an analysis of protein conformation in solution.

1. Introduction

Spectroscopic techniques have been used over a number of years for examining the conformations of proteins in solution. Recent advances both in theory and instrumentation have made possible a much more critical examination of the solution structure of these complicated biological macromolecules. Thus, while the amount of information obtainable has increased considerably, the limitations of the approach have also become better understood. It would seem, however, that with a number of precautions, a semi-quantitative analysis is possible, at least in some favorable cases.

The theoretical analysis of the amide I and amide II infrared absorption bands of polypeptides by Miyazawa (1960) (also Miyazawa & Blout, 1961), as extended by Krimm (1962), together with the recent complete band assignment of polyglycine (Suzuki *et al.*, 1966), have provided a stronger basis for the correlation of band positions and frequency shifts with probable conformations and conformational changes. In the realm of ultraviolet frequencies, the availability of sensitive recording optical rotatory dispersion (ORD)

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and circular dichroism (CD) instrumentation with penetration down to 185 mμ has opened to examination the optical rotation bands associated with particular conformations of peptide bonds.

Each of the various spectroscopic techniques is subject to great uncertainty due to band overlaps and shifts; the simultaneous examination of a protein by several such techniques, however, should help to eliminate some of the ambiguities inherent in any single method. On this assumption, we have examined several proteins by optical rotatory dispersion in the visible and ultraviolet regions, ultraviolet circular dichroism and infrared spectroscopy, seeking a quantitatively consistent analysis of all the data. In this approach, three basic structures have been singled out: the α-helix, the β-conformation and the unordered structure. It seems that in at least some proteins a conformational analysis can be carried out as follows. (1) The ORD data between 600 and 300 mμ are corrected for the optical rotation contributions of bands in the 250–310 mμ region, calculated from the corresponding CD spectrum; (2) Moffitt & Yang (1956) a_0 and b_0 parameters are obtained from the corrected data, and analysed in terms of the three structures (Timasheff *et al.*, 1966*a*), using the corresponding intrinsic a_0 and b_0 parameters as described below; (3) the ORD and CD curves in the region between 185 and 260 mμ are resolved according to the structural contributions calculated in step 2; (4) the ORD curves are calculated from the decomposed CD data and compared with experimental results. The criterion of acceptability of the data analysis consists in the agreement between the calculated and experimental ORD curves, the agreement of the CD and ORD component curves with standard curves obtained with model compounds, and the consistency of the deduced structure with the position of the amide I band in the infrared spectrum. While such an analysis will result perforce in numerical fractions of the various structural components, quite obviously no great significance should be attached to the actual numbers obtained; they can serve, however, as qualitative indicators of the conformational composition of a protein. Such an analysis is therefore of greatest value in assessing the relative amounts of the various structures present in a protein and in rationalizing the changes which occur in protein conformation with changes in environment.

A similar analysis can be carried out using the Schechter & Blout (1964) (Carver *et al.*, 1966*b*) A_{193} and A_{225} parameters. The two sets of parameters are linearly related; for $\lambda_0 = 212$ mμ,

$$\begin{aligned} A_{193} &= 0{\cdot}51\ a_0 - 4{\cdot}06\ b_0 \\ A_{225} &= 0{\cdot}51\ a_0 + 2{\cdot}97\ b_0. \end{aligned} \tag{1}$$

Therefore, essentially identical results can be expected whether the Schechter–Blout or Moffitt–Yang parameters are used. The b_0 parameter is particularly suitable to the estimation of the helical content, since this parameter is known

to be essentially insensitive to non-conformational effects (Tanford *et al.*, 1960; Carver *et al.*, 1966*b*), as is also

$$(A_{193}-A_{225}) = -7{\cdot}03\ b_0, \text{ at } \lambda_0 = 212\ m\mu.$$

ORD analyses of antiparallel chain β-structured polypeptides have resulted in zero or very small values of b_0 (Fasman & Blout, 1960; Imahori & Yahara, 1964; Ikeda *et al.*, 1964; Anufrieva *et al.*, 1965). Furthermore, since the b_0 of the unordered conformation is also small, b_0 becomes the parameter of choice for estimating the amount of α-helix. The choice of the a_0 intrinsic parameters is more difficult since this quantity is known to be affected by non-conformational effects, such as solvent and side chain interactions (Tanford *et al.*, 1960; Carver *et al.*, 1966*b*). In the present calculations, a_0 has been varied between 0 and +360 for the α-helix, −500 and −690 for the unordered structure and +400 and +700 for the β-conformation, using in each case the companion b_0 value reported for the particular a_0. The results give a surprisingly consistent qualitative picture. For example, for native β-lactoglobulin A, at pH 2, $a_0 = -140$, $b_0 = -63$ (after correction for the 270–300 mμ Cotton effects); the resulting conformational analysis gives: α-helix, 10–17%; unordered, 45–60%; β, 24–42%. These numbers indicate the type of results that can be expected at present. One can say that, in this protein, there is probably little α-helix, about half unordered and the rest β; furthermore, as will be shown below, the β-conformation is of the antiparallel type. In the following analysis the estimate of the β-conformation has been fixed at the higher value, i.e. 40%, since in the infrared spectrum of this protein the amide I band is dominated by the antiparallel chain β-structure (Timasheff & Susi, 1966).

In what follows, the analysis of β-lactoglobulin will be presented in detail, followed by a summary of results obtained with several other proteins. Since the described applications of circular dichroism and infrared spectroscopy are based on quite recent developments, it seems desirable first to sketch the basis for the analysis of protein conformations in terms of these two techniques.

In our experiments the i.r. spectra were obtained with a Beckman IR7 instrument, the ultraviolet ORD spectra principally with a Cary Model 60 spectropolarimeter, the ORD data in the visible and near ultraviolet region with a Rudolph 200S and a Durrum–Jasco ORD/UV-5 instrument, which was also used to determine the CD spectra.

2. Circular Dichroism

The electronic transitions of the peptide group give rise to a number of spectral bands in the wavelength region below 240 mμ. The strong rotatory power of these bands can be determined either from ORD Cotton effects or

from CD absorption bands. While the ORD technique has been widely used with ever increasing success (Carver *et al.*, 1966*a*,*b*), CD presents the advantage of having relatively narrow positive and negative bands which can be resolved with greater ease than the corresponding infinitely broad ORD bands. The ORD Cotton effects can be calculated band by band from the CD spectrum with the Kronig–Kramers transform (Moffitt & Moscowitz, 1959; Moscowitz, 1960,1962; Beychok & Fasman, 1964). The residue rotation at any given wavelength, λ, $[m']_\lambda$, is related to the ellipticity, $[\theta]_\lambda$, by the relation:

$$[m']_\lambda = \frac{3}{n_\lambda^2+2}\left\{\frac{R_k}{0{\cdot}696\times10^{-42}}\frac{\lambda_k^0}{\Delta_k^0}\frac{2}{\pi}\left[e^{-x^2}\int_0^x e^{t^2}\,dt - \frac{\Delta_k^\circ}{2(\lambda-\lambda_k^\circ)}\right]\right\} \quad (2)$$

where

$$R_k \approx 0{\cdot}696\times10^{-42}\sqrt{\pi}[\theta]_k^\circ\frac{\Delta_k^\circ}{\lambda_k^\circ}$$

where $x = (\lambda-\lambda_k^\circ)/\Delta_k^\circ$, λ_0 is the position of the Gaussian kth band, $[\theta]_k^\circ$ is its maximum ellipticity, Δ_k° is the half-width of the band at $[\theta] = [\theta]_k^\circ/e$, R_k is the rotational strength of the band and n_λ is the refractive index of the solution at the given wavelength. The sum of the Cotton effects should result in the experimentally observed ORD spectrum.

Using this approach, the CD spectra of synthetic polypeptides in the α-helical and random conformations have been characterized over the past five years (Holzwarth *et al.*, 1962; Brahms & Spach, 1963; Grosjean & Tari, 1964; Beychok & Fasman, 1964; Holzwarth & Doty, 1965; Velluz & Legrand, 1965; Townend *et al.*, 1966; Sarkar & Doty, 1966) and compared successfully with the corresponding ORD data. More recently, the CD spectrum of the antiparallel pleated sheet β-conformation has been examined in experiments on poly-L-lysine (Townend *et al.*, 1966; Sarkar & Doty, 1966) and silk fibroin (Iizuka & Yang, 1966); the poly-L-lysine spectrum in H_2O at pH 11·7 generates an ORD pattern (Townend *et al.*, 1966) in very good agreement with the experimentally observed one (Davidson *et al.*, 1966). The CD spectra of poly-L-proline in forms I and II and of the collagen triple helix are reported in this paper. The observed and calculated characteristic bands and their intensities are summarized in Table 1, together with the sources of the quoted values. From the several spectra reported so far, it would appear that the CD spectra of polypeptides in the α-helical and unordered (or random) conformations do not vary very seriously with the chemical nature of the side chains and solvents; the 208 mμ α-helix band is the one most sensitive to these variables (Carver *et al.*, 1966*b*). The CD band intensities of the antiparallel chain β-structure, on the other hand, seem to be strongly affected by the polarity of the medium; Iizuka & Yang (1966) have observed that in silk

TABLE 1. Circular dichroism of polypeptides in various conformations

Structure	Band position (mμ)	$[\theta']^a \times 10^{-3}$ (deg cm^2 decimole^{-1})	$R_k \times 10^{-40}$ (erg cm^2 rad)
α-Helix	221–222[b, c, d]	−30·4[b]; −30·3[c]	−22[b]; −16·9; −18·5[d]
	207[b, c, d]	−28·5[b]; −29·2[c]	−29[b]; −13·6[c]; −12·5[d]
	190–192[b, c, d]	55·0[b]; 52·5[c]	81[b]; 29·7[c]; 38·5[d]
Unordered	238[e]; 235[d]	−0·2[e]	−0·05[e]; −0·15[d]
	217[d, e]	small[b]; 2·4[e]	0·8[e]; 2·0[d]
	202[b]; 196[e]; 197[d]	−35·8[b]; −26·0[e]	−16·4[e]; −14·3[d]
Antiparallel-β in H_2O	217[c, f]	−14·3[c]; −16·5[b]	−10·7[c]
	195[c]	21·5[c]	14·1[c]
in SDS or MeOH	217[e, f, g]	−8·7[e]; −6·8[f]; −6·0[g]	−6·6[e]
	197[e, g]	29·6[e]; 22·2[g]	12·1[e]; 24·2[g, h]
	190[e]	15·6[e]	4·1[e]
theoretical[h]	218; 198; 195	−; +; +	
Parallel-β (theoretical)[h]	216; 181	−; +	−4 to −7; 21–24
Poly-L-proline I in n-propanol[e]	236 (?)[e]	−4·0[e, j]	−1·7[e, j]
	214[e]; 216[i]	58·0[e, j]	28·4[e, j]
	200[e]; 203[i]	−29·5[e, j]	−18·2[e, j]
Poly-L-proline II in H_2O	221[d, e]; 216[i]	16[d]; 12·9[e]	5·0[d]; 6·5[e]
	207[d, e]; 202[i]	−40[d]; −34·8[e]	−33·0[d]; −32·2[e]
Calfskin collagen	223[e]	2·1[e]	0·9[e]
	198[e, i]	−22·0[e]	−13·3[e]
	188[i]	+	

[a] $[\theta'] = \frac{3}{n^2 + 2}[\theta]$.

[b] Holzwarth & Doty (1965).

[c] Townend *et al.* (1966); the values of R_k reported in this paper were not corrected for refractive index.

[d] Carver *et al.* (1966*a*).

[e] This paper.

[f] Sarkar & Doty (1966).

[g] Iizuka & Yang (1966).

[h] Pysh (1966).
Pysh (1967).
Values uncorrected for refractive index.

fibroin the 197 mμ positive peak increased and the 217 mμ negative peak decreased when the polarity of the solvent was changed from 50 to 93% methanol; a similar decrease of the 217 mμ peak was reported by Sarkar & Doty (1966) when poly-L-lysine was caused to assume a β-conformation by dissolving in 0·06 M sodium dodecyl sulfate (SDS) rather than by heating a pH 11–12 aqueous solution to 40–50°C.

The CD spectrum of poly-L-lysine in 0·12 M SDS obtained by us from 185 to 245 mμ is shown in Fig. 1. Again the reported (Sarkar & Doty, 1966) decrease of the 217 mμ band is evident; in addition the absorption in the

195 mμ region has increased, and the 196 mμ band appears to be split into two bands, centered around 190 and 197 mμ. Pysh (1966) has calculated the ultraviolet optical properties of the parallel and antiparallel β-conformations, with the conclusion that the antiparallel form should have ultraviolet (u.v.) absorption and positive CD bands at 195 and 198 mμ. Comparison of the calculated and observed CD band positions seems reasonable in view of both the experimental difficulties encountered at the very low wavelengths and the assumptions inherent in the theory, such as neglect of side chain contributions and solvent effects. Furthermore, the i.r. spectrum of the same sample, shown in Fig. 4, confirms its nature as an antiparallel chain β-form, as will be shown below. The calculations of Pysh (1966) have also added a new important criterion for distinguishing between parallel and antiparallel pleated sheet β-structures: in the parallel form, the strong positive dichroic band is present at 181 mμ rather than 195–198 mμ.

The ORD spectrum of poly-L-lysine in 0·12 M SDS was calculated from the data of Fig. 1 using the Kronig–Kramers transform (equation 2) and the results are shown in Fig. 1. The three CD bands give rise to two positive and one negative Cotton effects. Their sum is represented by the solid line and is compared with the experimental ORD data of Sarkar & Doty (1966) above 195 mμ, shown by the circles. The agreement is found to be quantitative. Both curves are characterized by a shallow trough at 231 mμ, a peak at 203 mμ and cross-over points at 243, 225 and 194–195 mμ. The calculated curve, in addition, predicts a trough at 187 mμ. It seems significant that the positions of

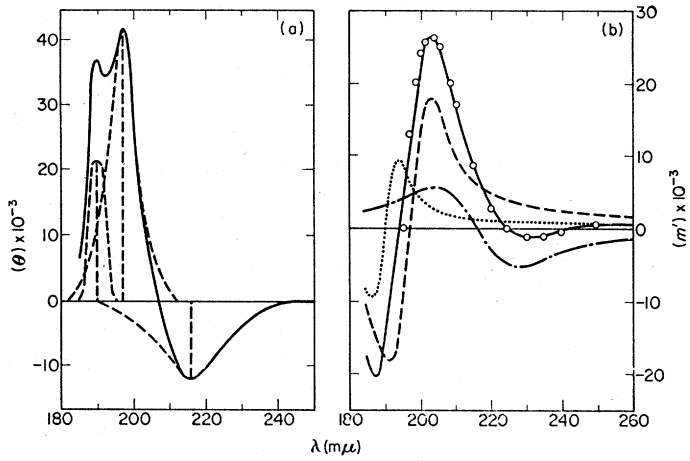

FIG. 1. Circular dichroism of poly-L-lysine at pH 7·8 in 0·12 M SDS. (a) Solid line, experimental curve; broken lines: individual bands. (b) Results of Kronig–Kramers (K–K) transformation showing the three individual Cotton effects and their summation; the circles above 195 mμ are the experimental ORD data of Sarkar & Doty (1966).

both the experimental and the calculated peaks in the presence of SDS have been shifted down by 2–3 mμ from the one observed (Davidson *et al.*, 1966; Sarkar & Doty, 1966) and calculated (Townend *et al.*, 1966) for the same polypeptide in H_2O at pH 11–12, after heating to 50°C.

These observations point to the great caution with which a CD analysis of protein structure must be undertaken. Thus, while the characteristic parameters of the α-helix and random conformation bands do not seem to vary too drastically, both the positions and intensities of antiparallel β-structure bands are a function of the nature of the solvent: in the case of silk fibroin, the intensity of the 197-mμ band increases by 50% between 50 and 93% methanol (Iizuka & Yang, 1966). It can be expected, then, that the antiparallel-β 217-mμ band will be very weak in the highly nonpolar interior of a globular protein, while the 196 mμ band will be strong.

The unordered structure of a protein is more difficult to define. This term embraces a vast variety of conformations present in a structural region devoid of continuous order along the polypeptide chain. Due to steric requirements, however, this structure is limited to a restricted number of possible conformations and can, in approximation, be regarded as a type of conformation with a characteristic CD spectrum; thus, the observed poly-L-lysine (Fig. 2) and calculated polyglutamic acid CD spectra in the unordered form (Carver *et al.*, 1966*a*) are in quite good agreement (Table 1). The CD spectrum of poly-L-lysine at pH 7·8, where it is in the unordered conformation, is shown in Fig 2. It is characterized by a strong negative band at 196 mμ, a weak positive band at 217 mμ, and very weak negative absorption centered at

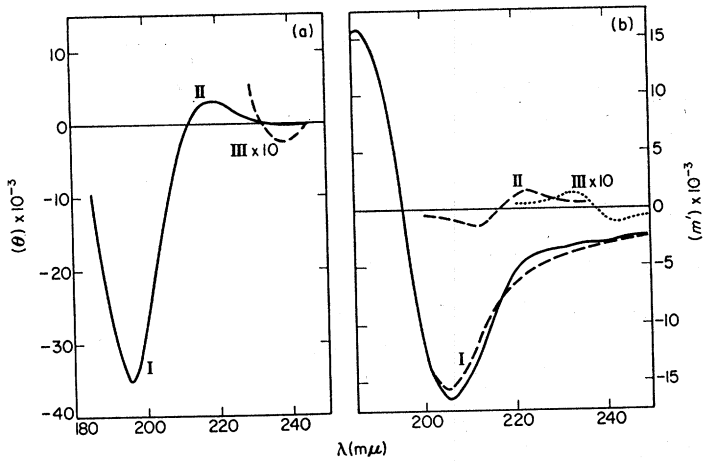

FIG. 2. Circular dichroism of poly-L-lysine at pH 7·8 in water. In (a) the bands are marked I, II, III. (b) ORD curve calculated by the Kronig–Kramers (K–K) transformation, showing the three separate Cotton effects and their summation.

about 238 mμ, and is in good agreement with that reported by Holzwarth and Doty between 205 and 225 mμ. The electron transitions of the first two bands have been discussed before (Holzwarth & Doty, 1965). The weak negative absorption between 230 and 245 mμ is unexpected (Carver *et al.*, 1966*a*) and may be quite likely only the tailing off of the strong negative 196 mμ band (E. S. Pysh, private communication). Kronig–Kramers transformation of the resolved bands results in the ORD spectrum shown on the right-hand side of Fig. 2. This curve is in quite reasonable agreement with those reported for unordered polyglutamic acid (Blout *et al.*, 1962; Yang, 1965; Iizuka & Yang, 1966; Carver *et al.*, 1966*a*). It would appear, then, that the observed ORD and CD curves for polypeptides in the α-helical and unordered conformations might be usable in an analysis of protein spectra. The difference curve between the experimental protein spectrum and the sum of the estimated α-helical and unordered contributions would correspond to the residual conformational and chromophoric side chain spectra. Comparison of the last curve with spectra given by polypeptides in various β or other known conformations in different media could serve to identify the residual structure.

Three other important known conformations are the two types of poly-L-proline helix and the collagen triple helix. The CD spectra of polyproline I and II† between 185 and 250 mμ were determined and are shown in Fig. 3 with the parameters summarized in Table 1. The corresponding data for collagen are shown in Fig. 9 and Table 1. Polyproline I is characterized by a strong positive absorption maximum at 214 mμ and a weaker negative absorption maximum at 198 mμ. Polyproline II gives a pattern with a strong negative absorption peaking at 205 mμ and a weak positive absorption maximal at 230 mμ. It is well known that in any method with overlapping bands, whether it is spectroscopic or moving boundary, when bands are close together, the resulting overlap may distort the apparent contributions of the individual bands and give maxima away from the true centers of the bands. In analysing ORD data for polyproline II, Carver *et al.* (1966*a*) concluded that the corresponding CD spectrum must have a strong negative band at 207 mμ and a weaker positive one at 221 mμ. Their CD measurements on poly-L-proline II between 215 and 260 mμ are indeed essentially identical with ours (Fig. 3) and consistent with their calculated curve. We analysed our data by reflecting the low wavelength side of the spectrum about 207 mμ (shown by the dotted lines in Fig. 3). The difference between this and the experimental data results in a Gaussian positive band at 221 mμ. The positions and rotational strengths of these bands are compared with those calculated by Carver *et al.* (1966*a*) in Table 1 and found to be in reasonable agreement. The CD spectrum of polyproline I was analysed similarly in terms of two strong bands

† This study was carried out in collaboration with Dr. W. F. Harrington.

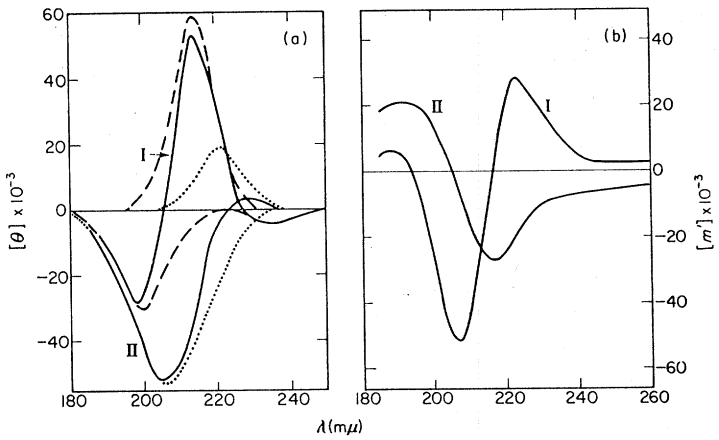

FIG. 3. (a) Circular dichroism of poly-L-proline I (in n-propanol) and II (in water), with the individual bands shown by the dashed and dotted lines. (b) The ORD patterns calculated by Kronig–Kramers transformation (K–K). The calculated residue rotations for form I have not been corrected for refractive index.

and one weak band as listed in Table 1. The positions and magnitudes of the bands were established by reflecting about 200 mμ the low wavelength side of the negative band and about 214 mμ the high wavelength side of the positive absorption. The resolution into bands was considered reasonable when both gave Gaussian shapes (taking the negative absorption above 228 mμ into account) and their sum was exactly the experimentally observed spectrum. Pysh (1967) has predicted theoretically the CD spectra of poly-L-proline. While for both forms of polyproline the positions of the bands are somewhat displaced from the calculated values, their relative energies and relative strengths are in reasonable agreement with theory. The ORD curves, calculated from the CD of both forms, are shown on the right-hand side of Fig. 3. The positions of the peaks, troughs and cross-over points are in excellent agreement with experimental literature values (Blout *et al.*, 1963; Bovey & Hood, 1966; Fasman, 1966; Carver *et al.*, 1966*a*; W. F. Harrington, & N. V. Rao, unpublished. R. Townend & S. N. Timasheff, unpublished, while the amplitudes of the peaks and troughs are quite consistent with those observed in identical solvents.

Summarizing, it appears that, with a great deal of caution, CD can be used to greater advantage than ORD for the elucidation of polypeptide and protein conformations. The various characteristic structures give rise to sets of positive and negative bands which are distinct in position. In the case of ORD, on the other hand, the trough at 232 mμ, which has been regarded (and is still used frequently) as a criterion for the presence of the α-helix, is also found in the ORD spectrum of the antiparallel chain β-structure (Davidson *et al.*,

1966; Sarkar & Doty, 1966; Iizuka & Yang, 1966). Furthermore, a small trough can also be observed in unordered structures (Yang, 1965; Carver *et al.*, 1966*a*). It is true that the amplitude of this trough varies greatly with conformation; however, the mere fact of its presence cannot be used for diagnostic purposes. The 205 mμ trough, which characterizes a disordered (random) structure, is also found in the ORD spectrum of collagen and polyproline II; the latter, of course, also has a strong peak at 223 mμ, thus affording a means of distinction between the two conformations. Finally, it should be remembered that two such distinct structures as the collagen triple helix (Blout *et al.*, 1963) and the unordered polypeptide conformation (Blout *et al.*, 1962) give strikingly similar ORD and CD spectra.

3. Infrared Spectroscopy

Infrared spectroscopy in the region of absorption of amide I and amide II bands has been used for many years to investigate the conformation of polypeptides and fibrous proteins in oriented and unoriented films. The vast amount of accumulated experimental data has made possible an empirical correlation between band positions and conformations.

More recently, a theoretical analysis by Miyazawa (1960) (see also Miyazawa & Blout, 1961) has provided a basis for these correlations. The theory was extended by Krimm in 1962. For ordered polypeptide and protein configurations, vibrational coupling between neighboring peptide groups causes the amide I and amide II bands to split into a number of branches. The frequencies of the strongest branches of amide I bands arising from various configurations is given in Table 2. (The polarization of the bands is omitted, since globular proteins in solution cannot be oriented.) The spectra of native globular proteins, however, are of greatest interest when obtained

TABLE 2. Prominent amide I frequencies for various conformations

Conformation	Solid films[a]	Proteins in H_2O[b]	Proteins in D_2O[b]	Poly-L-lysine D_2O[b]
α-Helix	1650 cm^{-1}	1652	1650	1635
Unordered	1658	1656	1643	1645
Antiparallel-chain β-structure	1632 (s) 1685 (w)	1632 (s) 1690 (w)	1632 (s) 1675 (w)	1611 (s) 1680 (w)
Parallel-chain β-structure	(1632)	(1632)	(1632)	
ν_0	1658	1661	1654	1646

[a] From Miyazawa (1960); Miyazawa & Blout (1961); Krimm (1962).
[b] H. Susi, S. N. Timasheff & L. Stevens, unpublished.

in aqueous medium. Such measurements involve serious complications: H_2O has a strong absorption band centered at 1650 cm^{-1} coinciding with the amide I band. In order to circumvent this difficulty, spectra are usually obtained in D_2O which is transparent in this frequency region. Dissolution in D_2O, however, results in deuteration of the peptide group and in corresponding band shifts, as has been observed for synthetic polypeptides (Lenormant *et al.*, 1958; Susuki *et al.*, 1966). A study of polypeptides and three model proteins (myoglobin, α-helix; α_s-casein, unordered; β-lactoglobulin,† β-conformation) in films, Nujol suspension of crystals, D_2O solution and H_2O solution (Timasheff & Susi, 1966; H. Susi, S. N. Timasheff & L. Stevens, unpublished) has made possible amide I band assignments to various conformations in H_2O and D_2O. These are summarized in Table 2. The strongest component of the α-helix band remains invariant in all media; a shift of 15 cm^{-1} toward lower frequencies is observed for the unordered conformation when H_2O is replaced by D_2O (with concomitant deuteration of the peptide groups); a smaller downward shift occurs in the weak component of the amide I band for the antiparallel chain β-structure; the position of the strong component for the antiparallel chain β-band apparently remains unchanged for proteins whether in film, H_2O or D_2O solution. Synthetic polypeptides are not necessarily typical of a given configuration, as shown by the frequencies listed in the last column of Table 2 for poly-L-lysine in D_2O solution. The frequencies observed in H_2O and D_2O solution have been discussed by H. Susi, S. N. Timasheff & L. Stevens, unpublished, in terms of the approach of Miyazawa (1960) and Krimm (1962). The apparent insensitivity of some strong amide I branches to deuteration in the peptide groups is explained by equal but opposite changes of the numerical values of v_0 and the interaction constant D_1'.

Typical amide I bands observed with various conformations in D_2O and H_2O solution are shown in Fig. 4. The i.r. spectrum of poly-L-lysine in 0·12 M SDS in D_2O is typical for the antiparallel chain β-structure. A strong band is present at 1617 cm^{-1}, a much weaker one at 1680 cm^{-1}. The shift of the strong band to 1617 cm^{-1} (proteins in D_2O: 1632 cm^{-1}; poly-L-lysine in D_2O: 1611 cm^{-1}) in this highly perturbed medium (~37% w/v non-aqueous) points to the danger of transferring frequency values from one related system to another. In the case of poly-L-lysine, the β-conformation receives additional stabilization energy from hydrophobic side-chain interactions (Davidson *et al.*, 1966). The fact that antiparallel chain β-conformations are not all identical is strikingly evident from the i.r. spectra of aggregating partly denatured proteins; for example, in the methanolic denaturation of β-lactoglobulin, in which the protein is undergoing a native → helical

† Infrared spectra of β-lactoglobulin as a film have shown this protein to have much antiparallel chain β-structure; the same is true in H_2O or D_2O.

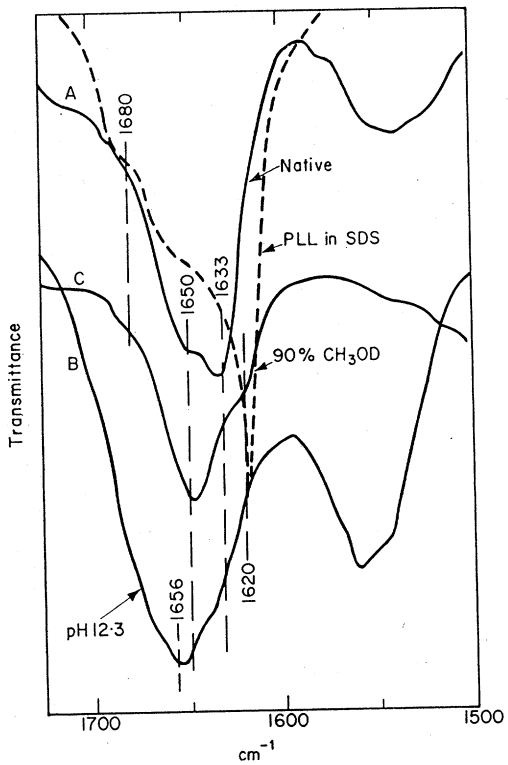

FIG. 4. Infrared spectra of poly-L-lysine and β-lactoglobulin, obtained by differential methods with the help of ordinate scale expansion. Dashed line, poly-L-lysine in 0·12 M SDS in D_2O solution. Solid line, β-lactoglobulin A. Curve A, native β-A in H_2O solution; curve B, alkali denatured β-A in H_2O solution; curve C, CH_3OD denatured in a 90% CH_3OD–10% D_2O mixture.

transition (Tanford *et al.*, 1960; Tanford & De, 1961), a band appears at 1616 cm^{-1} (S. N. Timasheff, H. Susi and L. Stevens, unpublished) at concentrations at which the protein aggregates (H. Inoue & S. N. Timasheff, unpublished), while only a normal 1632 cm^{-1} β-structure band is observed at lower concentrations at which no aggregation occurs; thus, at an intermediate methanol concentration, the i.r. spectrum of this protein contains bands both at 1632 and 1617 cm^{-1}, as well as at 1675 cm^{-1}, indicating the simultaneous presence of two anti-parallel chain β-structures of somewhat different nature.

As an example of protein spectra, the amide I bands of native β-lactoglobulin in H_2O, alkali denatured β-lactoglobulin in H_2O (pH 12·3), and CH_3OD denatured β-lactoglobulin in a CH_3OD–D_2O mixture are shown in Fig. 4. The three patterns have absorption maxima at 1632, 1656 and 1649 cm^{-1} respectively. The weak band at 1616 cm^{-1} in the methanol

denatured sample reflects the aggregation mentioned above. The positions of the bands in the three cases are quite consistent with the presence of considerable, or predominant, amounts of antiparallel chain β, unordered, and α-helical structures respectively. While an unequivocal assignment of structures from these spectra is dangerous, the fact that, under identical conditions, the ORD (Timasheff *et al.*, 1966*a*) and CD (see below) spectra point to the same conclusion renders these interpretations quite plausible. Furthermore, when alkaline denaturation is carried out in D_2O rather than H_2O, the amide I band is shifted to 1643 cm^{-1}, supporting the conclusion that, under these conditions, the protein is predominantly unordered (H. Susi, S. N. Timasheff & L. Stevens, unpublished).

Using the band assignments of Table 2, a number of proteins in D_2O solution and Nujol suspensions have been examined by i.r. spectroscopy in the amide I region. The results for some selected proteins are summarized below and compared with the conclusions drawn from their CD spectra.

4. Conformation of Individual Proteins

(a) *β-Lactoglobulin*

The i.r. spectra of β-lactoglobulin in three different conformations have been discussed above, while a detailed ORD study has been reported previously (Timasheff *et al.*, 1966*a*). The CD spectra of β-lactoglobulin A (β-A) in the 185 to 250 mμ (native and various states of denaturation) and 270–310 mμ (native) regions are shown in Figs. 5 and 6. In the peptide group transition region, the spectrum of the native protein is characterized by negative absorption between 245 and 204 mμ, maximal at 216 mμ, with a shoulder at 210 mμ and positive absorption at lower wavelengths with a peak at 193 mμ. While the negative part of the spectrum remains essentially unchanged between pH 1 and 7·5, the positive band shifts slightly toward lower wavelengths between pH 5 and 7·5, where the visible region rotation becomes more negative (Timasheff *et al.*, 1966*b*); at pH 9·0, the entire spectrum below 215 mμ is displaced to lower wavelengths, while at pH 9·9 the absorption becomes considerably stronger in the negative region, the curve develops a broad maximum between 210 and 215 mμ and the cross-over point shifts down to 199 mμ. These shifts seem to reflect the reversible conformational transition that occurs between pH 6·5 and 10 (Tanford *et al.*, 1959; Tanford & Nozaki, 1959; Tanford & Taggart, 1961; Timasheff *et al.*, 1966*b*). Above pH 10, β-lactoglobulin undergoes irreversible changes (Pantaloni, 1965). At pH 11, the negative CD band has doubled in intensity from pH 9, and the peak has shifted to 204 mμ, with a marked shoulder at 215 mμ. It is noteworthy that the spectrum of β-A with all disulfide bonds broken by *S*-sulfonation (Pèchère *et al.*, 1958) is essentially identical with the above pH 11

spectrum of the protein in which the disulfide bridges are presumably still intact. Both the i.r. and ORD spectra of β-A at the same pH indicate the presence of much unordered structure, while the b_0 constant remains unchanged from neutral pH; furthermore, a tyrosine residue (one of four per chain), previously unavailable to chemical modification, becomes reactive with cyanuric fluoride (M. J. Gorbunoff, unpublished). The CD spectrum, after one hour exposure of β-A to pH 13, is strongly negative below 230 mμ; this is consistent with the expected disruption of structure and probable changes in covalent bonds; simultaneously, the i.r. spectrum indicates a predominance of unordered conformation (cf. Fig. 4, pH 12·3) and the a_0 and b_0 constants shift to values close to those observed in 8 M urea (Timasheff *et al.*, 1966*a*). The disruption of the structure above pH 12 is not instantaneous: in β-B, the CD spectrum changes with time toward one more characteristic of an unordered structure, while the second unavailable tyrosine residue ionizes fully after 1 h at pH 13 (M. J. Gorbunoff, unpublished).

The CD spectrum of β-A in acidic methanol is shown in the inset of Fig. 5. The spectrum is typical of a predominantly α-helical structure. Estimates from

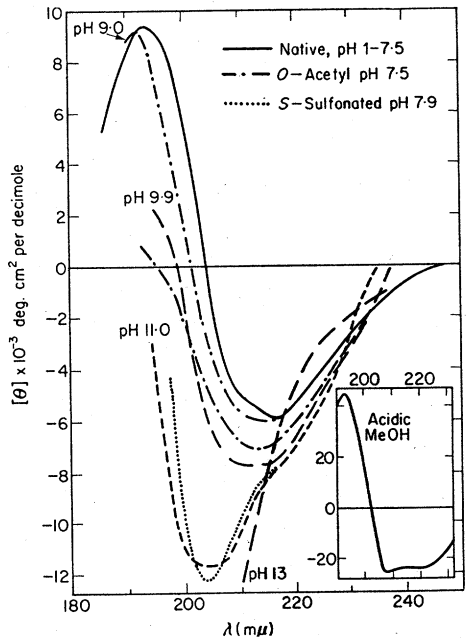

FIG. 5. Circular dichroism of β-lactoglobulin A in the native state and various states of denaturation. Conditions for the individual curves are shown on the figure and discussed in the text.

ORD (Timasheff *et al.*, 1966*a*) and i.r. (S. N. Timasheff, H. Susi and L. Stevens, unpublished) indicate the presence of about 70% α-helix, with the rest being equally divided between antiparallel β and unordered conformations. The observed intensities at 193, 208 and 221 mμ are consistent with such a configurational composition.

The CD spectrum of the native protein was subjected to the three structure analysis described above. Before starting this analysis, however, it was necessary to correct the rotations above 300 mμ for contributions from the Cotton effects between 270 and 310 mμ (Timasheff *et al.*, 1966*b*). This contribution was calculated from the corresponding CD spectrum, which is shown on the top of Fig. 6. The aromatic region CD spectrum of β-A consists

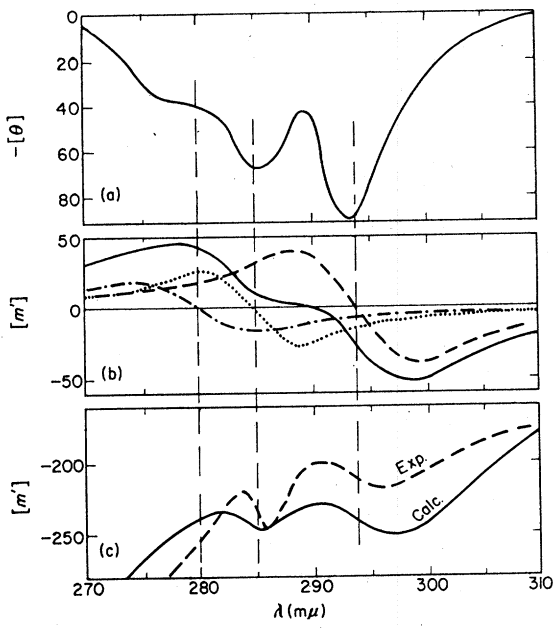

FIG. 6. (a) Circular dichroism of β-lactoglobulin A in the aromatic chromophore region; (b) calculated contribution to ORD; (c) comparison of calculated and experimental ORD curves.

of three principal negative bands at 293, 285 and 280 mμ with ellipticities of -80 to -40 deg cm^2 decimole^{-1}, i.e. two orders of magnitude smaller than the values associated with the 190–230 mμ bands. There appear to be also some strongly overlapping positive and negative bands below 275 mμ which are evidenced by a generally irreproducible weak, quite noisy, CD spectrum and a noisy ORD pattern between 275 and 260 mμ. The positions of the three principal bands are consistent with a mutually non-random arrangement of tryptophan residues, being at the same wavelengths as CD bands observed in

polytryptophan films (R. Townend, S. N. Timasheff & G. D. Fasman, unpublished). The lower wavelength absorption probably reflects order in tyrosine (Beychok & Fasman, 1964) and cystine (Beychok, 1965) residues. Reaction of β-A at pH 7·8 with acetylimidazole results in the blocking of all tyrosine residues (M. J. Gorbunoff, unpublished). The CD spectrum of the derivative, however, is quite similar to that shown in Fig. 6, supporting the conclusion that the observed bands are not caused by tyrosine residues, but rather by tryptophans. The CD spectrum of the derivative below 230 mμ, shown in Fig. 5, shows a change of structure from the native, but still within the region of reversibility.

Kronig–Kramers transformation of the CD spectrum of native β-A between 270 and 310 mμ gave the ORD Cotton effects shown in the middle of Fig. 6; superposition of their sum on the strong background negative rotation stemming from the far ultraviolet bands results in the solid line, shown in the lower part of the figure. This curve is in reasonable agreement with the experimental one, shown by the dotted line. The ORD spectrum appears to consist of either one positive or two negative Cotton effects, while in actuality it is the sum of three; this demonstrates the danger of interpreting aromatic ORD Cotton effects simply by inspection.

It is evident that Cotton effects in the 270–300 mμ region can affect the apparent values of Moffitt–Yang or Shechter–Blout parameters (Fasman *et al.*, 1965; Fasman *et al.*, 1964; Kronman *et al.*, 1965). In the case of β-A, the contribution of the 280, 285 and 293 mμ bands to rotation varies from $-0{\cdot}9°$ to $-18{\cdot}0°$ between 578 and 313 mμ. The corresponding uncorrected and corrected rotations are plotted in Fig. 7 in the Moffitt–Yang form. The best straight line drawn through the experimental points at the six highest wavelengths shows a strong deviation at 334 and 313 mμ. Correction of the

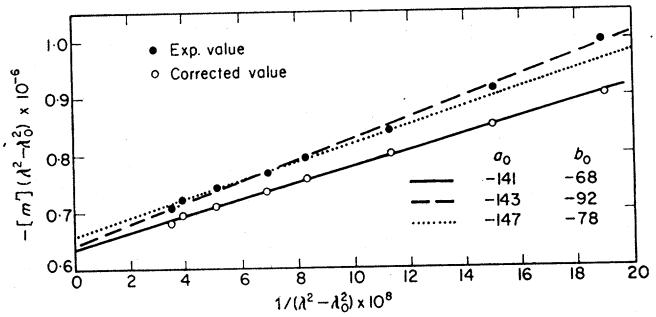

FIG. 7. Moffitt–Yang plot of the ORD of native β-lactoglobulin A between 313 and 578 mμ. ●, Experimental points; ○, same points corrected for contribution from CD bands of Fig. 6. Broken line, best straight line through experimental points; dotted line, best straight line through points at highest six wavelengths; full line, straight line through corrected points.

data point by point for the aromatic bands brings all the points on a straight line. The resulting a_0 and b_0 values for the straight line through the corrected points, the line through the highest wavelength points, and the best straight line through all the uncorrected points are listed on the figure. It is evident that lack of correction affects a_0 much less than b_0; thus, the b_0 values calculated previously for this protein using the last method (Herskovits *et al.*, 1964) are too negative by about 20%. An average of the corrected b_0 values for native β-A gives −60 to −65, leading to the conclusion, discussed above, that this protein contains between 10 and 17% α-helix.

The three-component analysis was carried out as described above with a_0 and b_0 values of −140 and −63. The most likely composition (after comparison with the i.r. spectrum) was chosen as 10% α-helix, 40% antiparallel chain β and 50% unordered structure. (These are the values obtained using the α-helix and unordered conformation intrinsic parameters of poly-L-glutamic acid (Urnes & Doty, 1961) and the β-structure parameters of Ikeda *et al.* (1964).) Using this composition, the CD and ORD spectra were resolved into components, as shown in Fig. 8. In both ORD and CD, the contributions of the α-helical and unordered conformations were taken as 10 and 50%, respectively, of the corresponding poly-L-lysine spectra (Davidson *et al.*, 1966; Townend *et al.*, 1966; Fig. 2 of this paper). The difference spectra obtained by subtracting 10% α-helix plus 50% unordered from the

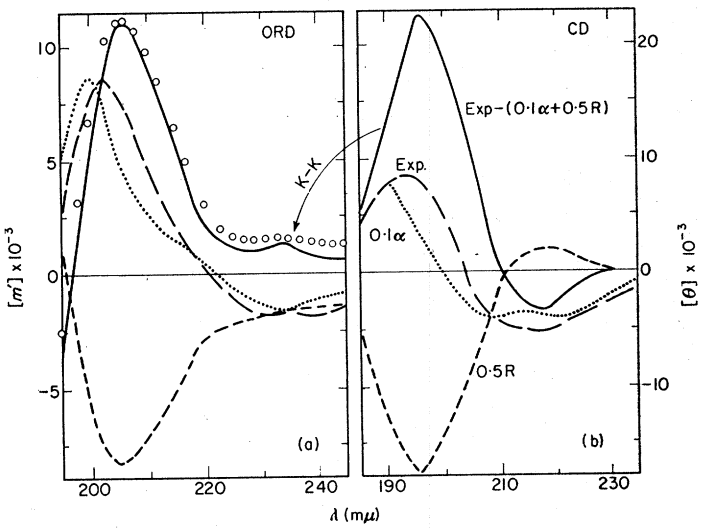

FIG. 8. Resolution of β-lactoglobulin A circular dichroism and optical rotatory dispersion patterns into conformational components, as described in the text. The circles in (a) represent the Kronig–Kramers (K–K) transformation of the solid line in (b) and are to be compared with the solid line in (a).

experimental curves are shown in each case by the solid lines. The CD difference spectrum has a strong positive band, maximal at 196 mμ, and a weak negative band, maximal at 217 mμ, i.e. the same positions as given by the antiparallel β-structure (see Table 1). The rotational strengths of these bands ($9{\cdot}9 \times 10^{-40}$ and $-1{\cdot}1 \times 10^{-40}$ erg cm^2 rad) are quite consistent with the location of this structure within the non-polar interior of the globular protein (cf. with Table 1). Kronig–Kramers transformation of the difference CD spectrum results in an ORD pattern almost identical with the experimental difference ORD spectrum. The ORD peak at 206 mμ is typical for an antiparallel β-structure (Blout & Shechter, 1963), while the absence of negative rotation in the 230 mμ region is not surprising in view of the very weak negative rotation found with poly-L-lysine in 0·06 M SDS (Sarkar & Doty, 1966) to 0·12 M SDS (Fig. 1) and positive rotation above 245 mμ. The small peak at 233 mμ in the experimental difference spectrum finds an extremely weak counterpart in the calculated curve. This, however, cannot rule out a contribution from aromatic side chain bands in this wavelength region.

The present analysis of the ORD and CD spectra of β-lactoglobulin A in terms of three structures, based on the a_0 and b_0 constants, demonstrates that this approach can be used to identify the presence of β-structure. Variation of the contents of unordered structure by $\pm 15\%$ resulted in difference spectra still consistent with β-structure; the amplitudes, however, of the antiparallel chain β-structure bands were inconsistent with the known intensities of polypeptides in the same conformations, i.e. either both were too low or both were too high. It would seem then that in a favorable case, such as β-lactoglobulin, such a conformational analysis can result in some degree of success; on the basis of these calculations, it seems reasonable to conclude that β-A contains probably a small amount of α-helix, about one-half unordered conformation and the rest antiparallel β-structure.

(b) *Other proteins*

The CD and i.r. spectra of several proteins of different conformation have been examined. The results are summarized in Table 3 and Figs. 9 and 10. These will be discussed in turn.

The CD spectrum of calfskin collagen† in pH 3·5, 0·3 M citrate buffer is shown in Fig. 9(a), and the parameters are summarized in Table 1. It is characterized by a strong negative band at 198 mμ and weak positive absorption at 223 mμ. The ORD spectrum calculated from the CD data has a minimum at 207 mμ and a crossover point at 198 mμ, in good agreement with the values reported by Blout *et al.* (1963). It is quite interesting to note that the collagen triple helix gives CD and ORD spectra very similar to those of the unordered conformation.

† A gift of Dr L. D. Kahn.

TABLE 3. Strongest amide I absorption of various *N*-deuterated proteins in D_2O solution[a]

Protein	Conditions	Frequency (cm^{-1})
Myoglobin	pD 7·0	1650
Lysozyme	6·4	1651 (~1630 shoulder)
β-Lactoglobulin	1–8	1632
	12·3	1643
	90% MeOH	1649
α_s-Casein B	pD 9·4	1643
Phosvitin	3–7·5	1650
Bovine serum albumin	2–6	1649
Bovine carbonic anhydrase	7·4	(1636)
	1·9	(1647)
Insulin	2 (fresh)	1654
Propionyl-CoA carboxylase	7·5	(1654)

[a] Values in parentheses represent preliminary data. Overlapping of different absorption bands can result in apparent absorption maxima which do not represent any single distinct configuration.

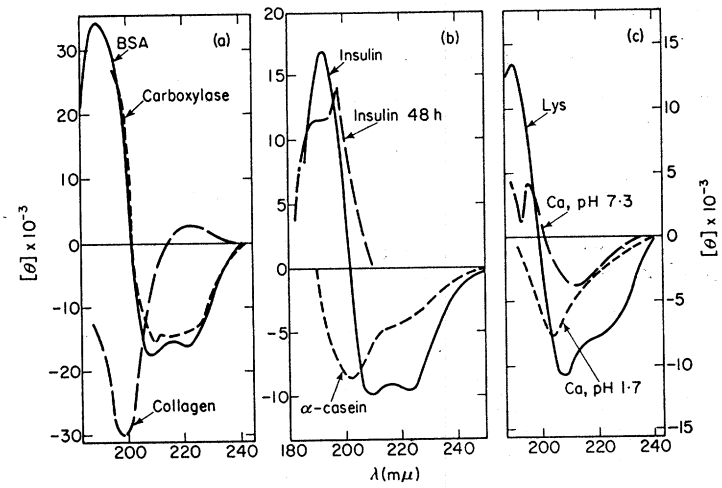

FIG. 9. Circular dichroism spectra of a number of proteins. (a) Bovine serum albumin, pH 5·8, water; collagen, pH 3·5, 0·3 M citrate; propionyl-CoA carboxylase, pH 7·0, 0·1 M tris. (b) α_s-Casein B, pH 7·5, 0·03 M NaF; insulin, pH 2, HCl–water, fresh and after 48 h at room temperature. (c) Lysozyme, pH 5·7, water; bovine carbonic anhydrase in water at pH 7·3 and 1·7.

Phosvitin† is a highly unusual protein in that 78% of its amino acids can carry a charge; of these 55% are phosphoserines, 17% are cationic, and 6% are carboxylic; only 10% of the amino acids are hydrophobic (Allerton & Perlmann, 1965). The dependence of its viscosity and optical rotation on pH (Jirgensons, 1958*a*,*b*) is typical for an unstructured polyelectrolyte. Perlmann & Allerton (1966) have examined its far ultraviolet ORD behavior. They found that as the pH's decreased from 7, a deep trough at 205 mμ decreases in amplitude and a second minimum appears at 232 mμ. The CD spectra at pH 3·4 and 6·6 are reported in Fig. 10. Both are marked by strong negative

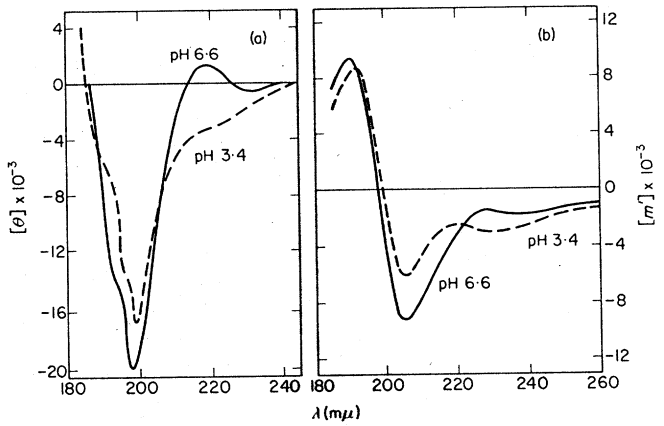

FIG. 10. (a) Circular dichroism of phosvitin at pH 3·4, water–acetic acid and pH 6·6, 0·1 ionic strength phosphate buffer. (b) ORD curves calculated by Kronig–Kramers (K–K) transformation; solid line, pH 6·6; broken line, pH 3·4, unordered and α-helical.

absorption, maximal at 199 mμ, with reproducible shoulders at 195 mμ (and 190 at 3·4). Above 210 mμ, the pH 6·6 spectrum exhibits a positive band at 214 mμ and a negative band at 233 mμ, just as polypeptides in the unordered conformation (see Fig. 2). Kronig–Kramers transformation gives an ORD pattern very similar to the experimentally observed one, including a very shallow trough at 235 mμ. Since at pH 3·4, the 232 mμ trough could reflect the presence of either β- or α-helical conformation, the corresponding CD pattern was examined as a combination of (a) unordered and α-helical, (b) unordered and antiparallel chain β, using the polylysine curves as standards. Kronig–Kramers transformation resulted in the ORD patterns shown by the broken and solid lines of the right-hand side of Fig. 10. It is evident that the shape of the experimental ORD curve (steep decrease in levorotation between

† This study was carried out in collaboration with Dr G. E. Perlmann and will be reported in detail elsewhere.

205 and 220 mμ, trough at 232 mμ) is reproduced only if the presence of about 10–15% α-helix is admitted, while β-structure formation does not account for the ORD pattern. Surprisingly, the amide I band in i.r. remained at 1650 cm^{-1} over the pD range studied. This position is inconsistent with the assignment of Table 2 for an unordered structure in D_2O. A possible explanation is that the strong repulsions between the charged side-chains of this protein impose additional constraints on the peptide linkages and cause a displacement in the positions of the corresponding i.r. bands. An alternate interpretation would consist in the assumption that phosvitin has the structure of a collagen-like triple helix; this would be consistent with the CD and ORD spectra, but, in view of the very low proline content (1·5%), it appears to be quite unlikely.

α_s-Casein is another protein which is considered to be unordered (Hipp *et al.*, 1952). The position of its (genetic variant B) amide I band at 1643 cm^{-1} in D_2O and 1656 cm^{-1} in H_2O at pH 9–10, $\Gamma/2 = 0{\cdot}1$, is consistent with this conclusion. The CD spectrum of the same protein at pH 7 in 0·03 M NaF is shown in Fig. 9(b). The negative band at 201 mμ with a strong shoulder at 220 mμ suggests again an unordered structure, mixed with α-helical or β. This protein, however, is subject to a complex pattern of aggregation (Swaisgood & Timasheff, 1967) and a detailed study of the effect of aggregation on its CD spectrum is in progress.

Insulin, in the native state, has an amide I band in the i.r. at about 1654 cm^{-1} in D_2O, which would be consistent with the predominance of a somewhat constrained α-helical structure. Its CD spectrum at pH 2, shown in Fig. 9(b), has the shape typical for an α-helix with peaks at 192, 209 and 223 mμ; this spectrum remains essentially unchanged up to pH 8·7. The amplitude of the CD bands corresponds to about 25% α-helix, a value consistent with the reported b_0 parameter (Urnes & Doty, 1961; Weil *et al.*, 1965). Furthermore, the amplitude of the 223 mμ band is in excellent agreement with the value found by Beychok (1965). In the case of insulin, however, Lindley & Rowlett (1955) (Lindley, 1955) have shown that, due to constraints imposed by S—S bonds, it is quite possible that insulin contains about 20% left-handed helix. The ORD and CD data would, of course, give only the excess right-handed over left-handed helix. Thus, the CD spectrum is not inconsistent with a helical content of 65% as deduced from the hypochromism of UV absorption near 190 mμ (Rosenheck & Doty, 1961).

After standing for two days at pH 2 and room temperature, the CD spectrum changed to one with positive maxima at 191 and 198 mμ. At acid pH, insulin forms fibrils (Waugh, 1954) which have a cross-β structure (Ambrose & Elliott, 1951). It would appear, then, that the observed transformation of insulin in solution from α-helical to β- conformation is a first step leading to the eventual fibril formation.

Bovine serum albumin (BSA) is considered to have about 50% α-helix (Urnes & Doty, 1961). Its amide I band is centered at 1649 cm^{-1} and the CD spectrum, shown in Fig. 9(a), has bands at 221, 208 and 190 mμ, all characteristic of α-helical conformation, their amplitudes being consistent with 45–50% of that structure. Other predominantly α-helical proteins have been reported to give also typical bimodal CD spectra above 200 mμ and a corresponding band at 191 mμ, e.g. myoglobin (Holzwarth & Doty, 1965) and myosin (Mommaerts, 1966).

The CD spectrum of propionyl-CoA carboxylase † shows a rather complex spectrum below 225 mμ (Fig. 9(a)), with some features characteristic of an α-helix contribution. The position of the amide I band at 1654 cm^{-1} is the same as found with insulin.

Bovine carbonic anhydrase was examined both in the native (pH 7·3) and acid denatured states (pH 1·7). At pH 7·3, the CD spectrum has broad negative absorption, centered at 216 mμ, while below 200 mμ the positive absorption is bimodal with maxima at 196 and about 190 mμ, as shown in Fig. 9(c). The amide I i.r. band is maximal at 1636 cm^{-1} with a shoulder at 1650 cm^{-1}. After acid denaturation this band is shifted to 1647 cm^{-1}, while the CD spectrum consists of a broad negative absorption region between 240 and 190 mμ, quite similar to alkaline denatured β-lactoglobulin. Comparison of these shifts with the ORD data of Rickli *et al.* (1964) and Coleman (1965) on the human enzyme suggests a transition from a native structure which contains considerable amounts of β and unordered components to an acid denatured one which is mostly unordered, with a small amount of α-helix. Such conformational compositions would account for the bimodal positive absorption in CD below 200 mμ in the native enzyme and the lack of positive absorption between 210 and 230 mμ at pH 1·7, as well as being consistent with the positions of the i.r. bands. In the case of carbonic anhydrase, however, particular care has to be exercised in an even semi-quantitative conformational analysis of CD bands because of the strong optical rotational bands related to order in chromophoric side chains (Myers & Edsall, 1965).

The CD spectrum of lysozyme, shown in Fig. 9(c), has negative absorption, maximal at 208 mμ, with a broad shoulder up to 230 mμ, in good agreement with the data reported by Sarkar & Doty (1966) above 200 mμ. Below 199 mμ, the absorption becomes positive and attains a peak at about 190 mμ. The amide I band in i.r. has a maximum at 1650 cm^{-1}, with a shoulder close to 1630 cm^{-1}, in good agreement with Hamaguchi (1964). The results are fully consistent with the known structure of lysozyme (Blake *et al.*, 1965), which consists of about one-third α-helix, 10–15% antiparallel chain β, and the rest of conformations which are grouped together under the general category of unordered.

† A gift from Dr J. Rabinowitz and D. Prescott.

This brief survey of a number of proteins of various structures can serve as an indication of the type of qualitative conformational analysis that may be carried out from a comparison of CD, ORD and i.r. spectra. The next, i.e. semi-quantitative step, must be undertaken only with a great deal of caution and should become more feasible with the availability of more data on standard, well-characterized model compounds.

REFERENCES

Allerton, S. E. & Perlmann, G. E. (1965). *J. biol. Chem.* **240,** 3892.

Ambrose, E. J. & Elliott, A. (1951). *Proc. R. Soc.* **A208,** 75.

Anufrieva, E. V., Bolotina, I. A., Volchek, B. Z., Illarionova, N. G., Kalikhevich, V. I., Korotkina, O. Z., Mitin, Yu. V., Ptitsyn, O. B., Purkina, A. V. & Estin, V. E. (1965). *Biofizika*, **10,** 918.

Beychok, S. (1965). *Proc. natn. Acad. Sci., U.S.A.* **53,** 999.

Beychok, S. & Fasman, G. D. (1964). *Biochemistry*, **3,** 1675.

Blake, C. C. F., Koenig, D. F., Mair, G. A., North, A. C. T., Phillips, D. C. & Sarma, V. R. (1965). *Nature, Lond.* **206,** 757.

Blout, E. R. & Shechter, E. (1963). *Biopolymers*, **1,** 565.

Blout, E. R., Schmier, I. & Simmons, N. S. (1962). *J. Am. chem. Soc.* **84,** 3193.

Blout, E. R., Carver, J. P. & Gross, J. (1963). *J. Am. chem. Soc.* **85,** 644.

Bovey, F. A. & Hood, F. P. (1966). *J. Am. chem. Soc.* **88,** 2326.

Brahms, J. & Spach, G. (1963). *Nature, Lond.* **200,** 72.

Carver, J. P., Shechter, E. & Blout, E. R. (1966*a*). *J. Am. chem. Soc.* **88,** 2550.

Carver, J. P., Shechter, E. & Blout, E. R. (1966*b*). *J. Am. chem. Soc.* **88,** 2562.

Coleman, J. E. (1965). *Biochemistry*, **4,** 2644.

Davidson, B., Tooney, N. & Fasman, G. D. (1966). *Biochem. biophys. Res. Commun.* **23,** 155.

Fasman, G. D. (1966). *Biopolymers*, **4,** 509.

Fasman, G. D. & Blout, E. R. (1960). *J. Am. chem. Soc.* **82,** 2262.

Fasman, G. D., Bodenheimer, E. & Lindblow, C. (1964). *Biochemistry*, **3,** 1665.

Fasman, G. D., Landsberg, M. & Buchwald, M. (1965). *Can. J. Chem.* **43,** 1588.

Grosjean, M. & Tari, M. (1964). *C.r. hebd. Séanc. Acad. Sci., Paris*, **258,** 2034.

Hamaguchi, K. (1964). *J. Biochem. Japan*, **56,** 441.

Herskovits, T. T., Townend, R. & Timasheff, S. N. (1964). *J. Am. chem. Soc.* **86,** 4445.

Hipp, N. J., Groves, M. L., Custer, J. H. and McMeekin, T. L. (1952). *J. Dairy Sci.* **35,** 272.

Holzwarth, G. & Doty, P. (1965). *J. Am. chem. Soc.* **87,** 218.

Holzwarth, G., Gratzer, W. B. & Doty, P. (1962). *J. Am. chem. Soc.* **84,** 3194.

Iizuka, E. & Yang, J. T. (1966). *Proc. natn. Acad. Sci., U.S.A.* **55,** 1175.

Ikeda, S., Maeda, H. & Isemura, T. (1964). *J. molec. Biol.* **10,** 223.

Imahori, K. & Yahara, I. (1964). *Biopolymers, Symp.* **1,** 421.

Jirgensons, B. (1958*a*). *Arch. Biochem. Biophys.* **74,** 57.

Jirgensons, B. (1958*b*) *Arch. Biochem. Biophys.* **74,** 70.

Krimm, S. (1962). *J. molec. Biol.* **4,** 528.

Kronman, M. J., Blum, R. & Holmes, L. G. (1965). *Biochem. biophys. Res. Comm.* **19,** 227.

Lenormant, H., Baudras, A. & Blout, E. R. (1958). *J. Am. chem. Soc.* **80,** 6191.

Lindley, H. (1955). *Biochim. biophys. Acta*, **18,** 194.
Lindley, H. & Rowlett, J. S. (1955). *Biochim. biophys. Acta*, **18,** 183.
Miyazawa, T. (1960). *J. chem. Phys.* **32,** 1647.
Miyazawa, T. & Blout, E. R. (1961). *J. Am. chem. Soc.* **83,** 712.
Moffitt, W. & Moscowitz, A. (1959). *J. chem. Phys.* **30,** 648.
Moffitt, W. & Yang, J. T. (1956). *Proc. natn. Acad. Sci. U.S.A.* **42,** 596.
Mommaerts, W. F. H. M. (1966). *J. molec. Biol.* **15,** 377.
Moscowitz, A. (1960). *In* "Optical Rotatory Dispersion", ed. by C. Djerassi, p. 150. New York: McGraw-Hill.
Moscowitz, A. (1962). *Adv. Chem. Phys.* **4,** 67.
Myers, D. V. & Edsall, J. T. (1965). *Proc. natn. Acad. Sci. U.S.A.* **75,** 4099.
Pantaloni, D. (1965). Doctoral Dissertation, University of Paris.
Pechere, J., Dixon, G. H., Maybury, R. H. & Neurath, H. (1958). *J. biol. Chem.* **233,** 1364.
Perlmann, G. E. & Allerton, S. A. (1966). *Nature, Lond.* **211,** 1089.
Pysh, E. S. (1966). *Proc. natn. Acad. Sci. U.S.A.* **56,** 825.
Pysh, E. S. (1967). *J. molec. Biol.* In press.
Rickli, E. E., Ghazanfar, S. A. A., Gibbons, B. H. & Edsall, J. T. (1964). *J. biol. Chem.* **239,** 1065.
Rosenheck, K. & Doty, P. (1961). *Proc. natn. Acad. Sci. U.S.A.* **47,** 1775.
Sarkar, P. K. & Doty, P. (1966). *Proc. natn. Acad. Sci. U.S.A.* **55,** 981.
Schechter, E. & Blout, E. R. (1964). *Proc. natn. Acad. Sci. U.S.A.* **51,** 695.
Suzuki, S., Iwashita, Y., Shimanouchi, T. & Tsuboi, M. (1966). *Biopolymers*, **4,** 337.
Swaisgood, H. E. & Timasheff, S. N. (1967). *Arch. Biochem. Biophys.* In press.
Tanford, C. & De, P. K. (1961). *J. biol. Chem.* **236,** 1711.
Tanford, C. & Nozaki, Y. (1959). *J. biol. Chem.* **234,** 2874.
Tanford, C. & Taggart, V. G. (1961). *J. Amer. chem. Soc.* **83,** 1634.
Tanford, C., Bunville, L. G. & Nozaki, Y. (1959). *J. Amer. chem. Soc.* **81,** 4032.
Tanford, C., De, P. K. & Taggart, V. (1960). *J. Amer. chem. Soc.* **82,** 6028.
Timasheff, S. N. & Susi, H. (1966). *J. biol. Chem.* **241,** 249.
Timasheff, S. N., Townend, R. & Mescanti, L. (1966*a*). *J. biol. Chem.* **241,** 1863.
Timasheff, S. N., Mescanti, L., Basch, J. J. & Townend, R. (1966*b*). *J. biol. Chem.* **241,** 2496.
Townend, R., Kumosinski, T. F., Timasheff, S. N., Fasman, G. D. & Davidson, B. (1966). *Biochem. biophys. Res. Commun.* **23,** 163.
Urnes, P. & Doty, P. (1961). *Adv. Protein Chem.* **16,** 401.
Velluz, L. & Legrand, M. (1965). *Angew. Chem. int. Ed.* **4,** 838.
Waugh, D. F. (1954). *Adv. Protein Chem.* **9,** 326.
Weil, L., Seibles, T. S. & Herskovits, T. T. (1965). *Arch. Biochem. Biophys.* **111,** 308.
Yang, J. T. (1965). *Proc. natn. Acad. Sci. U.S.A.* **53,** 438.